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Cell Growth in a Porous Microcellular Structure: Influence of Surface Modification and Nanostructures

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This study investigates the growth, morphology and cell viability of bone cells (osteoblasts) inside microcellular graphitic foam having an interconnected porosity. This type of substrate can be useful as a scaffolding material for tissue growth, but has not been adequately investigated. The influence of various surface treatments was studied: an inorganic hydrophilic coating (silica), an organic coating (collagen), and grafting of carbon nanotubes (CNT) have been reported. It is seen that all foams have acceptable biocompatibility. Silica and collagen coatings tend to have more cell growth along pore walls but do not have any significant influence on overall nuclear density or cell viability. On the other hand, nanotube attachment results in simultaneous increase of cell proliferation, density and viability. These results indicate that attaching carbon nanotubes on surfaces of future implants may provide a hierarchical nanostructure with increased biocompatible surface area for improved cell attachment.

Keywords: Tissue Engineering, Nanotube Attachment, Porous Carbon, Surface Coatings, Biocompatibility.

Porous materials infiltrated with bone cells can be very beneficial as future implants which can simulate in-growth of human bone and promote osteo-integration. Bioengineering of these substrates include designing and modifying new materials to promote and enhance bone cell growth, while remaining non-toxic to surrounding tissues. Recent studies have targeted a range of solids that can be manipulated to be porous such as silicon and various polymers.[1–3] Some have shown that surface roughness improves attachment.[4,5] Others indicate that coatings like collagen can increase bone formation,[6] and several additional surface modifications can improve cell adhesion[7] and related properties.

One material that is stated as being a biocompatible is graphitic carbon.[8] The same surface chemistry is presently available as porous microcellular foam.[9] Several surface modification techniques on these shapes have been developed for various materials applications,[10] but whether they facilitate bio-compatibility and cell growth was not known. Very recently, a few studies have appeared on the possible use of bare carbon foam[11] and Titanium-coated carbon foam[12] as cell implant materials, and they both indicate reasonable biocompatibility and absence of toxicity. At this time, a more detailed study is possible, which can identify possible surface modification approaches tailored for specific biological environments. Such an investigation will provide effective assessment and development of this material for specific implants. This study is aimed at addressing their interaction with bone cells, and the main goals are the following: (i) determine if the cellular graphitic foam facilitates osteointegration and aids in healthy stable bone growth with the differentiation of osteoblasts, and (ii) investigate the effects of various coatings on foam that may influence cell growth. Monitoring of cell morphology has been performed via fluorescence imaging and scanning electron microscopy, and information on cell viability was obtained through biological assays.

Microcellular carbon foam samples were obtained from Koppers Inc. in large blocks and then cut down to $1 \times 1 \times 0.2$ cm cubes. They were rinsed with Phosphate buffered saline (PBS) to remove any debris, and sterilized under ultraviolet light prior to the seeding of cells.

The foam samples were then placed in 24 well culture plates and adhered to the bottom of the well with double sided carbon tape to keep the foam submerged in media during initial incubation.

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14. ABSTRACT

This study investigates the growth, morphology and cell viability of bone cells (osteoblasts) inside microcellular graphitic foam having an interconnected porosity. This type of substrate can be useful as a scaffolding material for tissue growth, but has not been adequately investigated. The influence of various surface treatments was studied: an inorganic hydrophilic coating (silica), an organic coating (collagen), and grafting of carbon nanotubes (CNT) have been reported. It is seen that all foams have acceptable biocompatibility. Silica and collagen coatings tend to have more cell growth along pore walls but do not have any significant influence on overall nuclear density or cell viability. On the other hand, nanotube attachment results in simultaneous increase of cell proliferation, density and viability. These results indicate that attaching carbon nanotubes on surfaces of future implants may provide a hierarchical nanostructure with increased biocompatible surface area for improved cell attachment.

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Three types of surface coatings were investigated in this study:

(i) Inorganic hydrophilic coating (Silica): A nano-scale coating of Silicon dioxide (SiO_2) was deposited on the surface by plasma deposition. Details of this deposition process, as well as chemistry and morphology of the resulting nanolayer have been discussed in earlier publications.[13]

(ii) Organic macromolecular coating (Collagen): Collagen solution was purchased from Upstate, and diluted (0.4% solution of 3.75 mg/mL) in sterile phosphate buffered saline (PBS). The uncoated foam samples were immersed in the diluted collagen solution under UV light for approximately 20 min. After a one hour incubation at room temperature the collagen solution was removed and the samples were left to air dry for 1–4 hours in a sterile hood.

(iii) Nanotube attachment on surfaces to create a hierarchical template: A nano-hair type layer of strongly attached carbon nanotubes (CNT) was fabricated on these surfaces using a two-step process developed recently in this group.[14]

This involves activation of the foam surface with plasma-derived silica, followed by CVD deposition of CNT using Xylene-Ferrocene solution in a two-stage furnace. Typical morphology of the CNT-coated foam is shown in Figure 1.

Human fetal osteoblast cells (hFOB 1.19, ATCC, CRL-11372) were cultured in 25-cm^2 tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO_2 in air. A 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium was supplemented with 10% fetal bovine serum (FBS), and 0.3 mg/mL of G418 disulfate salt. Fresh media was added approximately every two days. When the osteoblasts were confluent, they were trypsinized with 0.25% trypsin diluted in PBS. To determine the cell density the cells were counted in a hemocytometer. The suspended cell solution was then centrifuged at 1000 rpm for 5 minutes and resuspended in fresh medium.

For all experiments that were conducted, 1.5 mL of hFOB suspension at a density of 1×10^6 cells/mL was placed in 24 well plates containing the uncoated and coated microcellular foam samples as well as an empty control well. Fresh medium was replaced on the second day of the incubation period, and the cells were incubated for a total of 72 hours.

Cell viability was measured using the CellTiter 96® AQueous One Solution assay from Promega. The solution contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)] and an electron coupling reagent (phenazine ethosulfate; PES). After the cells were grown for 72 hours, the MTS solution was added to each well. They were then incubated for one hour in which the media turned a reddish color. This solution was removed and placed in a 96 well plate and positioned in a plate reader to record the absorbance. The quantity of formazan product as measured by the amount of 490 nm absorbance and is directly proportional to the number of living cells in culture.

For cell morphology, after the incubation period, the medium was removed and the samples were rinsed twice with PBS. The cells were fixed with 2.5% glutaraldehyde in PBS, and then dehydrated through a series of ethanol dilutions. Following drying, the samples were mounted on a specimen holder with carbon tape then viewed at various magnifications on a JOEL JSM-7401 Field Emission Scanning Electron Microscope.

In order to study nuclear sizes and density of cells, fluorescence labeling was performed. After 72 hours the culture plates were rinsed twice with PBS. The cells were then fixed with 4% paraformaldehyde at room temperature

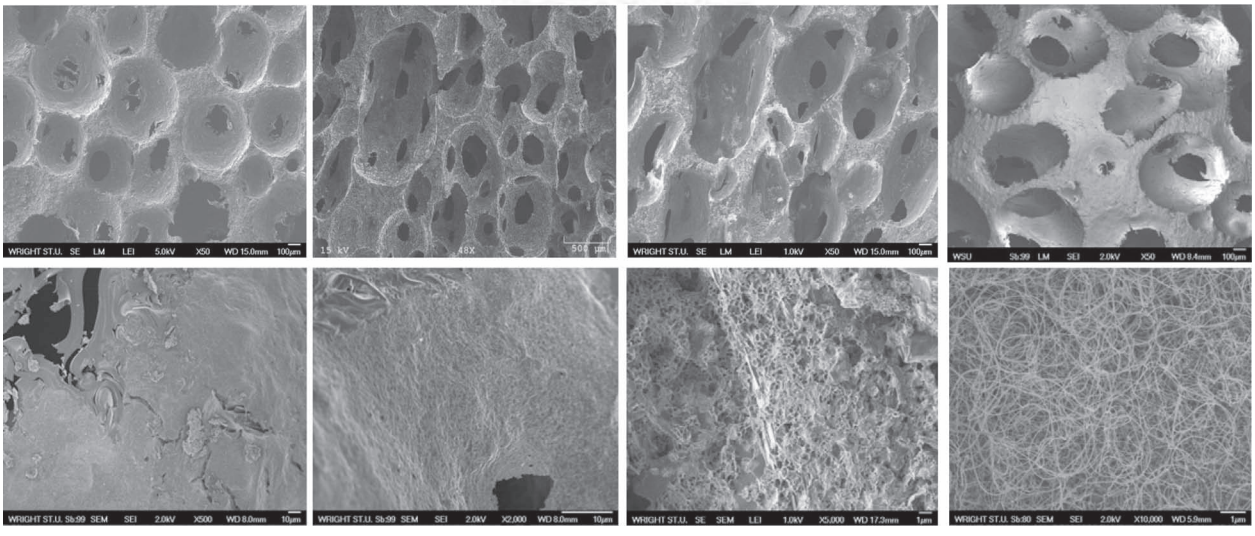

Fig. 1. SEM images of Uncoated foam (UC), Silicon Dioxide (SiO_2) coated foam, Collagen Coated (CC) foam, and Carbon Nanotube (CNT) Coated foam shown respectively. Images with Standard low magnification of 50× are in the upper level, while higher magnification pictures showing texture of pore walls are shown below.

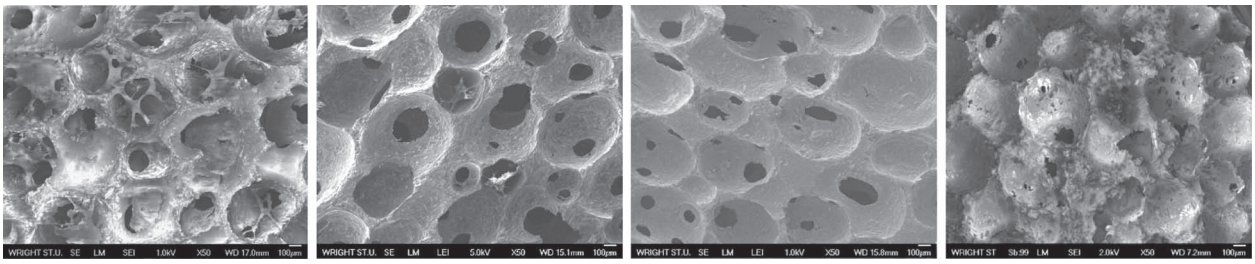

Fig. 2. SEM images of Uncoated foam (UC), Silicon Dioxide (SiO_2) coated foam, Collagen Coated (CC) foam, and Carbon Nanotube (CNT) Coated foam shown respectively. Pictures are of the cells grown on each of the coated foams for 72 hours.

for 10 minutes. The cells were rinsed with PBS and permeabilized with 0.1% Triton X-100 for five minutes. After rinsing with PBS, 1% BSA(Bovine Serum Albumine) solution was added for 30 minutes at room temperature. The actin filaments were then stained with Alexa Fluor 555-phallodin from Invitrogen for 20 minutes. The samples were then rinsed with PBS and a few drops of Prolong Gold Reagent with DAPI nuclear counter stain was added. After curing for 1 day at 2–6 °C in the dark, foam samples were then flipped upside down into a separate empty well so as to image the cells on the Olympus IX71 Inverted Fluorescent Microscope.

Figure 2 shows SEM images of osteoblasts cultured on various surfaces. On the uncoated foam image it can be seen that the cells spread well, and span across complete pores which are 500–600 micrometers in diameter. As can be seen in the fluorescence images in Figure 3, these structures are actually a network of cells. On the silicon dioxide coating, the cells formed tight clusters close to the pore walls, which is expected since these coatings are hydrophilic, and enhance wetting of the walls with the culture medium. The number of cell networks that span across open pores in the given incubation period is significantly reduced. The collagen coated (CC) foam had the typical porous network prior to incubation as seen in Figure 1, and these were seen to be completely covered with cells. However, none of the cells seemed to have multiplied enough to be spanning across the pores. For the carbon nanotube attached (CNT) foam, cell growth was more prolific in all respects. Cell walls were completely covered, and it can be seen from images such as Figure 2 that cell networks spanning across open pores was increased, noticeably more prevalent than seen in uncoated foam.

Using fluorescence microscopy, the cells on the foam were imaged with DAPI and TRITC filters which made the nucleus and actin fluoresce blue and red respectively. As stated earlier, this enabled in determination that structures spreading across pores were actually an entire network of cells.

Multiple images from the same sample were used to get estimates of average cell sizes and densities summarized in Table I. It must be noted that the images are two-dimensional representations of curved three-dimensional surfaces. Therefore, the actual quantitative values are not expected to be accurate. However, comparison of these numbers obtained on identical foam geometries can provide some very important insight about how the different surface modification techniques influence cell growth. The biological implication of average cell size is not clear at this point, but nuclear density may be a good indicator of how well they divide. Another observation was that among all surfaces, images on the collagen and carbon nanotube coated samples showed the most actin, which may be an indicator of stronger bond between the cells and foam.

The images from uncoated (UC), and carbon nanotube coated (CNT) foams are shown in Figure 3 for comparison. It is worth noting that the cell's actin filaments on the carbon nanotube coated foam (Fig. 3) are seen to be elongated and directional, most probably following the morphology of the underlying nanotubes. This indicates that the CNT structures may provide a suitable template that not only increases surface area for cell growth, but also may provide a directional template in future implant applications.

MTS results averaged over three or more asssays have been summarized in Table I along with cell sizes and

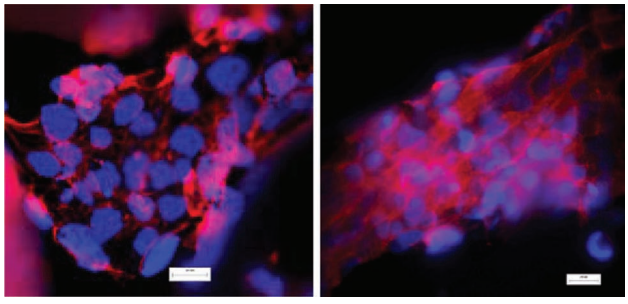

Fig. 3. Fluorescence images of uncoated (left), and carbon nanotube coated (right) foam samples.

Table I. Summary of cell morphology and viability MTS values (compared to control) and average cell parameters.

Sample type	Nuclear density ($\times 10^{-3}$ cells/μm^2)	Nuclear size (μm)	MTS (% control)
Uncoated foam	2.2	15.4	102.9
SiO_2 coated foam	2.8	12.6	92.4
Collagen coated foam	2.4	16.4	96.6
CNT attached foam	4.2	12.5	107.1

densities seen from fluorescent images. It must be noted that all samples yield MTS assays above 90% of control, implying acceptable biocompatibility. Silica and collagen-coated foams were seen in SEM to be adhering to the surface of pores in the foam. They appear to have marginally higher nuclear densities, but marginally lower MTS viability compared to uncoated foam. On the other hand, the CNT grafted foams are more enhanced in all aspects: they have increased adhesion to pore walls and increased cell network spanning on open pores as seen in SEM, significantly higher nuclear density as seen in Fluorescence imaging, along with some increase in mitochondrial activity as seen by MTS assays.

In summary, this study shows that it is possible to grow healthy and viable bone cells in microcellular carbon foam. Among different surface modification approaches studied, grafting of carbon nanotubes on the surface provides the best overall improvement. The simultaneous increase in nuclear density and cell viability may be attributed to the fact that nanotubes create a hierarchical network of increased biocompatible surface that can provide additional nucleation and growth sites for cells to thrive. There have been earlier reports of CNT dispersed in biological medium causing improvement of cell growth. This report is the first direct observation that the same benefit can also be seen in CNT strands strongly attached to a larger, more robust, substrate. This opens up the possibility of using nanotube-grafted implants for faster healing and integration at implant-tissue interfaces.

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